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Effect of flavophospholipol on conjugation frequency between *Escherichia coli* donor and recipient pairs *in vitro* and in the chicken gastrointestinal tract

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***Objectives*: To examine the ability of flavophospholipol to inhibit bacterial conjugation between *Escherichia coli* donor and recipient pairs *in vitro* and in day-of-hatch chickens.**

***Methods*: *In vitro* donor cultures were incubated in the presence of 0, 2, 4, 8, 16, 32 and 64 mg/L flavophospholipol during primary overnight mono-cultures only, secondary conjugation cultures only, or throughout primary and secondary cultures. Transconjugants were selected using oxytetracycline and nalidixic acid. Treatment groups A–G (n = 20) of day-of-hatch broiler chickens received 0, 2, 4, 8, 16, 32 and 64 g/ton flavophospholipol, respectively, in their feed throughout the experiment. On day 4, all treatment groups were given 0.25 mL of donor and recipient *E. coli* at 7.0 and 9.0 $\log_{10}$ cfu/mL, respectively. On day 10, the birds were euthanized and the caecal contents were cultured on selective medium (oxytetracycline and nalidixic acid).**

***Results*: A dose-dependent reduction in transconjugant populations was observed *in vitro* when flavophospholipol was present in the secondary conjugation culture. The susceptibility profiles of transconjugants obtained from *in vitro* studies were identical to the predicted profile of the donor and recipient combination. There was no significant difference ($P \geq 0.05$) in the number of transconjugants isolated from chickens among any of the flavophospholipol treatment groups when compared with the controls. The susceptibility profiles of chicken transconjugants suggested acquisition of naturally occurring plasmids.**

***Conclusions*: Flavophospholipol strongly inhibited conjugation *in vitro*, but did not prevent recipient *E. coli* from acquiring resistance determinants *in vivo*.**

Keywords: horizontal gene transfer, microbial drug resistance, growth promotion

Introduction

The emergence of multidrug-resistant microorganisms is a global problem that has arisen from widespread use of antimicrobials in both human and animal populations. The application of antimicrobials at subtherapeutic levels for disease prophylaxis and growth promotion in poultry and livestock is believed, by some, to increase selection for antimicrobial-resistant populations in the gastrointestinal tract of these animals.[1–3] The problem is exacerbated because this type of treatment is often applied to large herds or flocks, subsequently creating a large reservoir for antimicrobial-resistant bacteria.

A large, diverse population of facultative and strict anaerobic bacteria live in the gastrointestinal tract[4] and the bacterial genomes of these microorganisms make up the reservoir of potential resistance genes. Localization of resistance genes on mobile genetic elements such as broad-host-range plasmids, transposons and integrons facilitates horizontal transfer of genetic material between bacteria, providing a rapid means of dissemination at the molecular level.[5–8] Horizontal transfer of antimicrobial resistance is one of the most significant issues with regard to our ability to curtail the emergence of multidrug-resistant bacteria.

Flavophospholipol (also known as bambermycin, flavomycin and moenomycin) is a phosphoglycolipid antibiotic that has been approved for use as a feed additive, to improve nutritional performance and intestinal health, in poultry, swine and cattle.[9,10] It primarily acts against Gram-positive bacteria, but it has been shown to reduce shedding of *Salmonella* in experimentally infected broilers[10] and has also been shown to inhibit growth of Gram-negative bacteria carrying certain R plasmids. Inhibitory

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specificity against bacteria carrying some R plasmids may explain the apparent inhibition of conjugation *in vitro* and the decrease in antimicrobial-resistant enteric bacteria *in vivo*.[11–13] Flavophospholipol is the only known feed additive to have been described with the potential to reduce horizontal dissemination of resistance genes.[12] In the past 30 years of use, no transferable form of resistance against flavophospholipol has been definitively described, and cross-resistance to therapeutic antimicrobials has not be observed.[9,12]

Most of the *in vivo* studies, performed to date, have not specifically examined inhibition of conjugation by colonizing animals with well-characterized donor and recipient pairs. *In vitro* conjugation studies have shown that pre-incubation of the donor strain with flavophospholipol is necessary to inhibit horizontal transfer of resistance genes.[13] This observation suggested that inducible gene expression was required for the inhibitory effect to be observed.[13] If this holds true in the animal, then continuous feeding of flavophospholipol would be necessary to prevent the horizontal dissemination of resistance.

The objective of the present study was to determine the effect of flavophospholipol on conjugation between well-characterized *Escherichia coli* donor and recipient pairs *in vitro* and in day-of-hatch chickens.

Material and methods

E. coli donor and recipient strains

Two donor strains were used in the *in vitro* studies. Donor strain one (DS1) *E. coli* CVM828 serogroup 0139, fimbrial type F107, contains a 150 kb conjugative plasmid. The conjugation frequency with *E. coli* lab strain JM109 was previously determined to be 7.1×10^{-5} per recipient. *E. coli* DS1 is positive for a Tn*21* transposon and exhibits resistance to ampicillin, chloramphenicol, kanamycin, sulfamethoxazole, tetracycline and trimethoprim/sulfamethoxazole. Donor strain two (DS2) *E. coli* CVM1548 serogroup O149, fimbrial type K88, contains a 190 kb conjugative plasmid with a Tn*21* transposon. *E. coli* DS2 had a previously determined conjugation frequency of 5.3×10^{-4} per recipient with JM109.[14] DS2 exhibits antimicrobial resistance to chloramphenicol, kanamycin, sulfamethoxazole and tetracycline. A nalidixic-acid-resistant recipient strain (RS1) was derived from a field strain that did not contain a plasmid and was negative by PCR for Tn*21*. RS1 was susceptible to all antimicrobials tested except chloramphenicol, cefalotin and nalidixic acid. In conjugation studies using RC1 as a donor, resistance to chloramphenicol and cefalotin could not be transferred.

Determination of antimicrobial susceptibility

The antimicrobial MICs were determined by broth microdilution according to methods described by the Clinical Laboratory Standards Institute (CLSI). Susceptibility testing was performed with the Sensititre® automated antimicrobial susceptibility system according to the manufacturer's instructions (Trek Diagnostic Systems, Westlake, OH, USA). NARMS panels (CMV7CNCD) for Gram-negatives were used in the Sensititre system; the following antimicrobials were assayed: amikacin, amoxicillin/clavulanic acid, ampicillin, cefoxitin, ceftiofur, ceftriaxone, cefalotin, chloramphenicol, ciprofloxacin, gentamicin, kanamycin, nalidixic acid, streptomycin, sulfamethoxazole, tetracycline and trimethoprim/sulfamethoxazole. The following ATCC strains were used as controls for antimicrobial susceptibility testing: *E. coli* 35218, *E. coli* 25922, *Staphylococcus aureus* 29213 and *Pseudomonas aeruginosa* 27853. Data were interpreted using CLSI breakpoints,[15] as described previously.[16]

Bacterial conjugation in vitro

The experimental design consisted of three groups. In group I, the primary monoculture consisted of donor cells (DS1 or DS2) grown overnight at 37°C in tryptic soy broth (TSB) containing 0, 2, 4, 8, 16, 32 or 64 mg/L flavophospholipol (Intervet, Inc., Millsboro, DE, USA) and 32 mg/L oxytetracycline followed, on day 2, by no treatment during the secondary conjugation co-culture. In group II, flavophospholipol (0, 2, 4, 8, 16, 32 or 64 mg/L) was present only during the secondary conjugation co-culture (day 2), but not during the primary culture (day 1). In group III, the concentrations used above were present during both the primary and secondary cultures. RS1 was grown overnight in 32 mg/L nalidixic acid for all experiments.

Bacterial conjugation in vivo

On day 2, primary donor and recipient mono-cultures were washed three times in TSB to remove residual antimicrobial treatments. The secondary broth conjugation co-cultures were made by mixing washed DS1 or DS2 cells (0.05 mL) from the overnight cultures with washed RS1 cells (0.5 mL) in 4.95 mL of fresh TSB. All conjugation combinations were incubated overnight at 37°C. Tenfold serial dilutions were spread plated and enumerated on tryptic soy agar (TSA) plates containing oxytetracycline (32 mg/L) and nalidixic acid (32 mg/L). Oxytetracycline was used to select for horizontal transfer of the plasmid, and nalidixic acid was used to counter-select against donor cells. Conjugation frequency per recipient was expressed by dividing the number of transconjugants by the initial number of recipients. Due to the complexity of the *in vivo* system the number of transconjugants obtained was expressed as cfu/mL rather than by conjugation frequency per recipient.

Experimental birds

Cobb × Ross day-of-hatch broiler chickens were obtained from a local hatchery. All chickens were placed in rearing pens at appropriate rearing temperature on clean pine shavings litter material. Chickens were provided water and a corn-soy based diet that met or exceeded National Research Council guidelines (1994) for *ad libitum* consumption. A United States Department of Agriculture Institutional Animal Care and Use protocol was followed during the study (IACUC no. 2004009).

In vivo experimental design

Day-of-hatch broiler chickens were separated into seven groups, A–G (20 birds/treatment). Group A received untreated feed. Groups B, C, D, E, F and G received medicated feed with 2, 4, 8, 16, 32 and 64 g/ton flavophospholipol, respectively. Analytical analysis of the flavophospholipol content in the feed was 86–88% of the theoretical level as analysed by Eurofins (Memphis, TN, USA). The feed treatments were provided for *ad libitum* consumption throughout the trials. Cloacal swabs were performed on all birds for 3 days prior to inoculation with donor and recipient strains to assure they were free of nalidixic-acid-resistant and oxytetracycline-resistant Gram-negative bacteria. Cloacal swabs were plated on MacConkey Agar (Difco, Detroit, MI, USA) containing nalidixic acid, oxytetracycline and both nalidixic acid and oxytetracycline. On day 4, 0.25 mL each of *E. coli* DS2 (donor) and *E. coli* 1578^{nal} (recipient) strains were administered via oral gavage to all birds at 10^7 and 10^9 cfu/mL, respectively. Birds were swabbed daily to determine the presence of the donor and recipient and transconjugant populations. All groups were euthanized on day 10.

Bacterial culture from in vivo specimens

To quantitatively measure donor, recipient and transconjugant *E. coli* populations the caeca were removed and 0.25 g of the caecal contents was placed in 2.25 mL of PBS. Ten-fold serial dilutions of caecal contents were performed in TSB and plated on MacConkey agar with oxytetracycline, nalidixic acid and both oxytetracycline and nalidixic acid. Culture plates were incubated at 37°C for 24 h. Following incubation donor, recipient and transconjugant colonies were counted and transconjugant stock cultures were saved for phenotypic and genotypic characterization.

Molecular analysis of transconjugants

All 80 *in vivo* and 10 *in vitro* transconjugants were examined by PFGE and compared with DS2 and RS1 to confirm clonality to the RS1 recipient strain. Chromosomal DNA was digested with *Xba*I (New England BioLabs, Beverly, MA, USA) according to the standard CDC protocol. Electrophoresis was performed using a CHEF Mapper XA Pulsed Field Gel Electrophoresis System (Bio-Rad Laboratories, Richmond, CA, USA) with 0.5× TBE running buffer (0.089 M Tris, 0.089 M boric acid, 0.001 M EDTA, pH 8.0). Run parameters were as follows: initial switching time of 5 s, final switching time of 35 s, 6 V/cm, 120° inclusion angle and a 20 h run time at 12°C. Gels were stained with ethidium bromide and band patterns were analysed using Molecular Analysis Fingerprinting Software, version 1.6 (Bio-Rad Laboratories, Hercules, CA, USA), using the unweighted pair group method arithmetic average (UPGMA).

To determine whether the Tn*21* transposon carried on the DS2 plasmid was transferred to the transconjugants PCR analysis was performed using Tn*21*-f and *intI1*-r primers as described previously.[16]

Statistical analysis

Differences in bacterial populations (cfu/mL) were statistically evaluated using the GLM procedure for one-way ANOVA (Sigma Stat, SPSS, Inc. Chicago, IL, USA). Replicate experiments performed *in vivo* on different dates were significantly different and were not pooled. Differences among mean values were considered significant at $P < 0.05$ level of significance.

Results

Effect of flavophospholipol on pure cultures

To determine possible combinations for donor and recipient pairs and because high levels of flavophospholipol may effect the growth of some Gram-negative bacteria, the effect of flavophospholipol on overnight growth of each strain was examined. Flavophospholipol treatment significantly reduced the DS1 overnight population at 8, 16, 32 and 64 mg/L and the DS2 overnight population at 16, 32 and 64 mg/L (Table 1). Flavophospholipol significantly reduced the recipient strain RS1 overnight population at 64 mg/L and the JM109^{nal} laboratory strain at 32 and 64 mg/L.

In vitro conjugation study

Because RS1 was a field strain, rather than a lab adapted strain, it was chosen as the recipient for all subsequent conjugation studies. Preliminary conjugation studies showed that flavophospholipol treatment of the overnight recipient RS1 culture (2, 4, 8, 16, 32 or 64 mg/L) had no effect on the conjugation frequency obtained from unmedicated conjugations (data not shown). Untreated control conjugation frequencies for donor stains DS1 and DS2 were 8.1×10^{-4} and 8.2×10^{-4} per recipient cell, respectively.

In group I, incubation of only the overnight culture with flavophospholipol, there was no significant difference in the number of transconjugants obtained between any of the flavophospholipol treatments and the untreated control. In study groups II and III, a dose-dependent reduction in the transconjugant populations was observed for both donor strains DS1 and DS2 (Figure 1a and b, respectively). The most significant inhibition occurred in group III experiments when flavophospholipol treatment was present during both overnight and conjugation cultures. No transconjugants were obtained at any flavophospholipol dose from DS1 group III experiments (Figure 1a). In DS2 group III experiments, the transconjugant populations at 2 and 4 mg/L flavophospholipol dropped to 2.9 $\log_{10}$ and 1.7 $\log_{10}$ cfu/mL, respectively, as compared with the untreated control at 8.66 $\log_{10}$ cfu/mL, and no transconjugants were observed at flavophospholipol dosages above 4 mg/L (Figure 1b).

In vivo conjugation study

DS2 was chosen as the donor strain for *in vivo* experiments. No nalidixic acid resistance was observed from cloacal swabs taken daily prior to inoculation with DS2 and RS1; however, up to two birds per group were colonized with a tetracycline resistance Gram-negative strain. Daily post-inoculation cloacal swabs showed that both DS2 and RS1 colonized all birds in all experiments and were present for the duration of each study. At the conclusion of the study both DS2 and RS1 were isolated from all

Table 1. Effect of flavophospholipol on *E. coli* donor and recipient overnight cultures ($\log_{10}$ cfu/mL)

	Flavophospholipol (mg/L)						
Isolate	0	2	4	8	16	32	64
DS1	8.91 ± 0.04	8.65 ± 0.04	8.65 ± 0.01	7.87 ± 0.04	7.13 ± 0.03	7.24 ± 0.02	6.8 ± 0.04
DS2	8.72 ± 0.06	8.62 ± 0.05	8.183 ± 0.0	7.99 ± 0.01	7.54 ± 0.02	7.292 ± 0.01	7.23 ± 0.04
JM109^{nal}	8.14 ± 0.05	8.35 ± 0.8	8.13 ± 0.4	8.22 ± 0.021	8.14 ± 0.08	7.78 ± 0.02	7.42 ± 0.05
RS1	9.09 ± 0.01	8.17 ± 0.08	8.10 ± 0.01	8.30 ± 0.04	8.2 ± 0.07	8.2 ± 0.26	7.29 ± 0.01

Mean ± SD of duplicate cfu/mL.

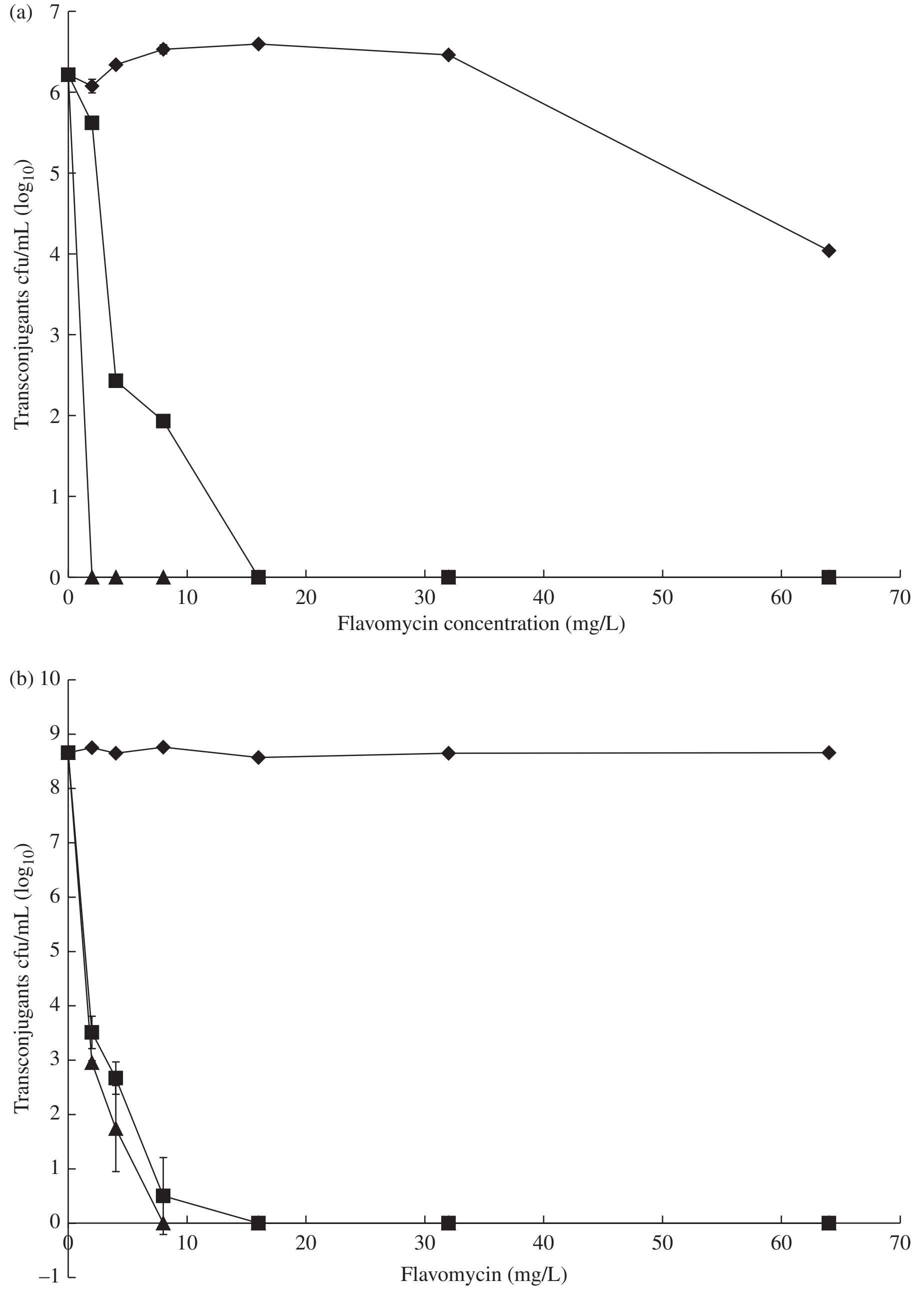

Figure 1. *In vitro* conjugation in the presence of flavophospholipol. (a) DS1/RS1; (b) DS2/RS1. Filled diamonds, group I, flavophospholipol treatment during the overnight culture only; filled squares, group II, flavophospholipol treatment during the conjugation culture only; filled triangles, group III, flavophospholipol treatment during both the overnight and conjugation cultures.

caecal samples at 7.0–8.0 $\log_{10}$ cfu/mL in all experiments. There was no significant difference in the number of transconjugants isolated from chickens among any of the flavophospholipol treatment groups when compared with the untreated controls ($P \geq 0.05$) (Table 2).

Susceptibility profiles and molecular analysis of transconjugants

A total of 5 transconjugants from each *in vivo* study group (n = 80) and 10 transconjugants from *in vitro* conjugations of DS2 and RS1 were genotypically analysed by PFGE and compared with DS2 and RS1 (data not shown). All transconjugants examined from *in vitro* and *in vivo* studies were clonal to RS1 as predicted. All the *in vitro* transconjugants were positive for the Tn*21* transposon as determined by PCR analysis (data not shown).

The susceptibility profile of transconjugants isolated from the *in vitro* conjugation of DS2 and RS1 demonstrated the expected susceptibility profile with resistance to six of the antimicrobials tested (cefalotin, chloramphenicol, kanamycin, nalidixic acid, sulfamethoxazole and tetracycline). The kanamycin resistance gene carried by DS2 did not confer cross-resistance to gentamicin.

The susceptibility profiles of the 80 transconjugants selected from the *in vivo* studies were much more complex. The number

Table 2. Total number of *E. coli* transconjugants ($\log_{10}$ cfu/mL) obtained from chicken caecal samples

		Experiment			
Flavophospholipol (g/ton)	Group	1	2	3	4
Control (0)	A	3.70 ± 0.85	4.11 ± 0.84	3.24 ± 0.87	3.72 ± 0.79
2	B	2.86 ± 1.18	4.39 ± 0.68	ND	ND
4	C	3.14 ± 0.64	4.26 ± 1.04	ND	ND
8	D	4.64 ± 1.74	4.06 ± 0.95	ND	ND
16	E	ND	ND	2.72 ± 0.66	3.96 ± 0.84
32	F	ND	ND	3.27 ± 0.60	4.34 ± 0.68
64	G	ND	ND	3.89 ± 0.72	3.46 ± 0.84

Values represent means from 15 birds ± SD.
ND, not determined.

of antimicrobials these isolates were resistant to ranged from 4 to 11, suggesting that RS1 acquired resistance traits from normal chicken gastrointestinal flora. The phenotypes correlated to the date each experiment was performed, rather than feed treatment (Table 3). Only 2 of the 80 transconjugants carried the identical susceptibility profile generated *in vitro* with DS2 and RS1. Four other transconjugants that also displayed resistance to 11 antimicrobials exhibited resistance to sulfamethoxazole, suggesting that they may have acquired the DS2 plasmid in combination with another plasmid. Seven (8.75%) of the transconjugants acquired the kanamycin, sulfamethoxazole and tetracycline combination carried by DS2; two of these transconjugants were in the untreated control of study three. One hundred per cent of the transconjugants exhibited tetracycline and nalidixic acid resistance because oxytetracycline and nalidixic acid were used for selection and counter-selection in all of the conjugations.

Forty-five of the transconjugants (56%) exhibited gentamicin resistance. Gentamicin resistance that did not confer cross-resistance to kanamycin was prevalent in the first two studies. In the third study, resistance to both gentamicin and kanamycin was present; this may have represented an aminoglycoside resistance gene not present on DS2 donor plasmid that conferred cross-resistance to both gentamicin and kanamycin. In the fourth study, all transconjugants were susceptible to gentamicin and 75% were susceptible to kanamycin. A total of 34% and 29% of the transconjugants in the study displayed resistance to ampicillin and amoxicillin/clavulanic acid, respectively.

Transconjugants from studies two and four carried fewer phenotypic resistance traits per isolate than studies one and three. In study three, 80% of the transconjugants displayed resistance to 10 or 11 of the antimicrobials tested. The susceptibility profiles exhibited by transconjugants isolated from untreated controls were also observed in the treated birds regardless of flavophospholipol dosage.

The Tn*21* transposon is very common in Gram-negative bacteria and was in present in 90%, 100% and 100% of the transconjugants from studies one, two and three, respectively. In study four, three of the five control group transconjugants carried Tn*21*; however, none from flavophospholipol treatment groups carried the Tn*21* transposon.

For the present study the number and molecular weight of plasmids carried by the transconjugants were not determined. It is possible that multiple plasmids were acquired *in vivo* making it difficult to determine whether flavophospholipol specifically inhibited conjugation between DS2 and RS1. The possibility of gene transfer by transducing phage was not examined. However, it is apparent that flavophospholipol was unable to inhibit acquisition, by the recipient, of mobile genetic elements conferring multidrug resistance.

Discussion

Although flavophospholipol is only approved for use in poultry at 2 g/ton, higher doses were used due to previous reports that flavophospholipol antimicrobial activity may be inactivated in the gut.[17] A product that inhibited bacterial conjugation and did not select for antimicrobial resistance would be a useful feed additive and might decrease dissemination of resistance genes in the gastrointestinal reservoir. To date flavophospholipol has not been shown to select for any known transferable mechanism of resistance.[12]

Poppe *et al.*[18] showed that horizontal transfer of a conjugative plasmid carrying multiple antimicrobial resistance genes could occur in the avian gastrointestinal tract without selection pressure. Under the conditions used in the present study, transconjugants were also readily obtained *in vivo* without selection pressure. This study attempted to inhibit conjugative transfer between known donor and recipient pairs using an antimicrobial growth promoter *in vivo*.

Flavophospholipol treatment of donor but not recipient broth cultures *in vitro* produced significant reductions in the number of transconjugants observed. A reduction of the transconjugant population was observed when the donor cultures were incubated in the presence of flavophospholipol during the conjugation portion of the experiments. A slightly greater reduction was observed when the overnight donor and conjugation cultures were incubated with flavophospholipol. This is consistent with previous results that suggested an inducible inhibition was responsible.[13] Flavophospholipol treatment only during the initial overnight incubation had no effect on the number of transconjugants produced as compared with the untreated controls when DS2 was used as the donor. Because the donor cells were washed to remove residual flavophospholipol prior to the conjugation culture and the conjugation culture was allowed to incubate overnight, the donor cells were probably able to overcome an induced

Table 3. Susceptibility phenotypes of *E. coli* transconjugants obtained from *in vivo* studies with day-of-hatch chickens

Flavophospholipol (g/ton)	Experiment 1			Experiment 2			Experiment 3			Experiment 4		
		Tn*21*	*n*		Tn*21*	*n*		*Tn21*	*n*		*Tn21*	*n*
Control	CEF-CHL-GEN-NAL-TET	+	1	CEF-CHL-GEN-NAL-TET	+	5	CEF-CHL-CIP-NAL-STR-TET	+	1	CEF-CHL-NAL-TET	–	2
	AMP-CEF-CHL-NAL-TET	–	1				AMC-AMP-CEF-CHL-FOX-GEN-KAN-NAL-STR-TET	+	2	CEF-CHL-KAN-NAL-TET	+	2
	CEF-CHL-GEN-NAL-STR-TET	+	2				AMC-AMP-CEF-CHL-FOX-GEN-KAN-NAL-STR-SUL-TET	+	2	AMC-AMP-CEP-CHL-KAN-NAL-TET	+	1
	AMP-CEF-CHL-GEN-NAL-STR-TET	–	1									
2	CEF-CHL-KAN-NAL-TET	+	1	CEF-CHL-GEN-NAL-TET	+	5	ND			ND		
	CEF-CHL-GEN-NAL-STR-TET	+	2									
	AMP-CEF-CHL-GEN-NAL-STR-TET	+	1									
	AMC-AMP-CEF-CHL-FOX-KAN-NAL-SUL-TET	+	1									
4	CEF-CHL-NAL-TET	+	2	CEF-CHL-GEN-NAL-STR-TET	+	1	ND			ND		
	CEF-CHL-GEN-NAL-STR-TET	+	1	AMC-AMP-CEF-CHL-GEN-NAL-STR-TET	+	4						
	AMP-CEF-CHL-GEN-NAL-STR-TET	+	1									
	AMC-AMP-CEF-CHL-GEN-NAL-STR-TET	+	1									
8	CEF-CHL-KAN-NAL-TET	+	3	CEF-CHL-GEN-NAL-STR-TET	+	5	ND			ND		
	CEF-CHL-KAN-NAL-SUL-TET	+	**2**									
16	ND			ND			CEF-CHL-NAL-KAN-TET	+	1	CEF-CHL-NAL-TET	–	3
							AMC-AMP-CEF-CHL-FOX-GEN-KAN-NAL-STR-TET	+	4	CEF-CHL-NAL-STR-TET	–	2
32	ND			ND			CEF-CHL-NAL-STR-TET	+	1	CEF-CHL-NAL-TET	–	5
							AMC-AMP-CEF-CHL-FOX-GEN-KAN-NAL-STR-TET	+	3			
							AMC-AMP-CEF-CHL-FOX-GEN-KAN-NAL-STR-SUL-TET	+	1			
64	ND			ND			CEF-CHL-NAL-STR-TET	+	1	CEF-CHL-NAL-TET	–	2
							AMC-AMP-CEF-CHL-FOX-GEN-KAN-NAL-STR-TET	+	3	CEF-CHL-KAN-NAL-TET	–	2
							AMC-AMP-CEF-CHL-FOX-GEN-KAN-NAL-STR-SUL-TET	+	1	CEF-CHL-NAL-STR-TET	–	1

ND, not determined; AMC, amoxicillin/clavulanic acid; AMP, ampicillin; CEF, cefalotin; CHL, chloramphenicol; GEN, gentamicin; KAN, kanamycin; NAL, nalidixic acid; STR, streptomycin; SUL, sulfamethoxazole; TET, tetracycline.
The profile given in boldface was identical to the expected profile generated from conjugation between donor DS2 and RS1.

inhibitory effect. In addition, daughter cells produced during the overnight incubation would never have been exposed to flavophospholipol. During preliminary experiments performed with only a 2 h conjugation incubation time as previously described,[13] very few transconjugants were produced in untreated controls and none was produced in the flavophospholipol-treated groups. Consequently, the 2 h broth incubation was extended overnight.

It has been reported that high levels of flavophospholipol can affect the growth of cells that are normally intrinsically resistant.[17] A 100-fold reduction in cell growth was observed for DS1 from the overnight culture incubated with 64 mg/L flavophospholipol. The reduction in the transconjugant population obtained from 64 mg/L flavophospholipol-treated DS1 may reflect the lower number of donor cells in the subsequent conjugation inoculum.

The transconjugants isolated from *in vitro* conjugations using DS2 all exhibited the predicted susceptibility phenotype with resistance to six antimicrobials. Acquired resistances to kanamycin, sulfamethoxazole and tetracycline were the phenotypic markers that suggested the DS2 plasmid had been horizontally transferred. The phenotypic resistance to kanamycin displayed by DS2 did not confer cross-resistance to gentamicin.

The most significant observation of the present study was that flavophospholipol did not prevent recipient *E. coli* from acquiring naturally occurring resistance determinants *in vivo*. It has been suggested that flavophospholipol may alter the structure of fimbriae such that an effective mating pair bridge may not occur.[13] The results from the *in vitro* studies suggest that the effect of flavophospholipol was on the donor but not on the recipient *E. coli*.

In the chicken caeca there was no significant difference in the number of transconjugants observed between any of the flavophospholipol treatments and the untreated controls run on the same date. However, the susceptibility profiles of the chicken transconjugants analysed suggest that plasmids present in the native microflora may have been transferred alone or in combination with the DS2 plasmid. The multidrug susceptibility profiles correlated to the date the birds were obtained from the local hatchery and not the flavophospholipol treatment.

Only 2.5% of the chicken transconjugants possessed a susceptibility phenotype identical to that obtained from *in vitro* conjugations. Only four additional transconjugants, which also displayed resistance to 11 antimicrobials, exhibited resistance to the kanamycin–sulfamethoxazole–tetracycline phenotype provided by DS2. Sulfamethoxazole appeared to be the best indicator that DS2 may have transferred its plasmid. Resistance to sulfamethoxazole was only exhibited by 8.75% of the chicken transconjugants. Since no selection pressure was used during the *in vivo* experiments, the DS2 plasmid may have sustained point mutations, deletions or recombination events that could have changed the phenotypic susceptibility profile. However, this was not observed among the 10 transconjugants examined from *in vitro* conjugations.

Gentamicin resistance was prevalent among the chicken transconjugants isolated *in vivo*. This was not surprising since gentamicin is present as a preservative in some poultry vaccines. Resistance to ampicillin and amoxicillin/clavulanic acid was not exhibited by DS2 and was likely provided by the normal flora. Tn*21* was highly prevalent, 76.25%, among the chicken transconjugants. If only six of the transconjugants acquired the DS2 plasmid, it is likely that Tn*21* was acquired from the normal flora.

Because the donor and recipient strains colonized the birds so well, it is surprising that the donor phenotype was not isolated more frequently. A number of factors could explain this. It is possible that the transconjugants obtained from transfer of the DS2 plasmid were not as fit as transconjugants that acquired naturally occurring plasmids. It is also possible that mutations or recombination events may have occurred masking the identity of markers used in the study. Further molecular characterization of the plasmids, transposons and integrons would be necessary to completely rule out these possibilities. It is possible that the low percentage acquisition of the donor phenotype was due to inhibition by flavophospholipol; however, the donor phenotype was largely absent in the untreated control groups as well.

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Transparency declarations

None to declare.

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